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## **Platelet Depletion Impairs Host Defence to Pulmonary Infection with *Pseudomonas aeruginosa* in Mice**

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### **Key Summary**

Platelets play a protective role in gram negative infections, as depletion of circulating platelets leads to enhanced severity and mortality in mice infected with *P. aeruginosa*.

\* These authors contributed equally to this work

### **AUTHORSHIP**

RA, SP, AB, MN, CP were involved with the conception, hypotheses delineation, and design of the study. RA and BGO contributed equally in data acquisition, analysis and interpretation. SA, SC, MN, SP were involved with acquisition of the data, analysis and interpretation of the data. RA wrote the article. All authors were involved with manuscript revision prior to submission. There are no conflicts of interest to declare.

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**AT A GLANCE SUMMARY**

*P. aeruginosa* infection of the lungs induces neutrophil recruitment, platelet recruitment and release of platelet derived pro-inflammatory mediators. Mice experimentally depleted of platelets infected with *P. aeruginosa* demonstrate decreased neutrophil recruitment combined with increased total bacterial load and increased mortality rates.

**ABSTRACT**

Platelets have been implicated in pulmonary inflammatory cell recruitment following exposure to allergic and non-allergic stimuli, but little is known about the role of platelets in response to pulmonary infection with *Pseudomonas aeruginosa* (*P. aeruginosa*). In this study, we have investigated the impact of the experimental depletion of circulating platelets on a range of inflammatory and bacterial parameters, and their subsequent impact on mortality in a murine model of pulmonary infection with *P. aeruginosa*. *P. aeruginosa* infection in mice induced a mild but significant state of peripheral thrombocytopenia in addition to pulmonary platelet accumulation. Increased platelet activation was detected in infected mice through increased levels of the platelet derived mediators PF-4 and  $\beta$ -TG in bronchoalveolar lavage fluid (BALF) and blood plasma. In mice depleted of circulating platelets, pulmonary neutrophil recruitment was significantly reduced 24 hours' post infection, whilst the incidence of systemic dissemination of bacteria was significantly increased compared to non-platelet depleted control mice. Furthermore, mortality rates were increased in bacterial infected mice depleted of circulating platelets. This work demonstrates a role for platelets in the host response towards a gram-negative bacterial respiratory infection.

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## INTRODUCTION

*P. aeruginosa* is a gram negative, opportunistic bacterium, recognised as one of the major colonising species in the lungs of cystic fibrosis (CF) patients with chronic bacterial infections, hospitalised cancer patients and in infections associated with severe burns [1–3]. The importance of platelets in both the innate and adaptive inflammatory responses has been highlighted through the release of numerous preformed mediators from internal alpha ( $\alpha$ ) and dense granules. These mediators contribute to the inflammatory response through processes such as cell adhesion and migration, whilst also contributing to wound repair [4]. Indeed, several animal models of inflammation have described a critical role for platelets in inflammatory cell recruitment. These include eosinophil and lymphocyte recruitment in allergic inflammation, and neutrophil recruitment in models of acute lung injury (ALI), rheumatoid arthritis (RA) and cardiovascular diseases (CVD) [5–8]. Furthermore, pulmonary platelet accumulation has been observed in models of allergic inflammation and ALI following allergic and non-allergic insults respectively [9,10].

Evidence of platelet activation in infectious diseases has been well documented, with increases in the expression of platelet activation markers in patients admitted to hospital with numerous infectious diseases. These markers include increases in the incidence of platelet-monocyte and platelet-neutrophil complexes in the circulation, increased levels of surface P-selectin expression and increased granular secretion [2]. A number of patients with sepsis also present to the clinic with thrombocytopenia, which has been associated with a worse prognosis and increased mortality when compared to patients presenting with normal platelet levels. This further highlights a role for platelets in this condition [11–13].

Platelet activation associated with bacterial infections has been reproduced in several animal models of pulmonary bacterial infections including *Staphylococcus aureus* and *Klebsiella pneumoniae* induced sepsis [14,15]. In these models thrombocytopenia has been associated with enhanced pulmonary bacterial load and increased systemic infection [15,16]. Direct interactions between platelets and numerous bacterial pathogens, including *S. aureus*, *Helicobacter Pylori* and *P. aeruginosa*, have been reported both *in vitro* and *in vivo* [17]. These interactions have been associated with increased pulmonary platelet accumulation and reversible platelet adhesion [17], whilst bacterial derived toxins have also been reported to modulate platelet function [18]. Other research has reported that direct interactions between platelets and microorganisms leads to subsequent phagocytosis of *S. aureus*, *S. pneumoniae* and *P. aeruginosa* by platelets, as an additional method of removing the bacteria from the infected tissue [14]. Furthermore, it has been suggested that platelets also possess the capacity to augment the antimicrobial effects of other immune cells [19,20].

Clearly, in addition to their involvement in hemostatic and inflammatory processes, there are several mechanisms by which platelets may contribute towards host defence in response to invading pathogens. The aim of this study was to investigate the role of platelets in pulmonary infection with *P. aeruginosa* by studying the effect of platelet depletion on bacterial induced leukocyte recruitment, bacterial growth and the systemic dissemination of bacteria. We utilised a well-established murine model of chronic lung infection induced by the *P. aeruginosa* strain RP73. This strain has been previously reported to show histopathological lesions akin to those observed in CF patients with chronic infections [21,22], thus making this a clinically relevant strain to investigate in the context of chronic lung infections.

## METHODS

Further details of methodology present within the supplemental section.

### **Infection with *P. aeruginosa* embedded in agar beads and induction of platelet depletion in mice**

All animal studies were carried out under appropriate UK and Italian legislation. Male C57/Bl6 mice were anaesthetised and inoculated with *P. aeruginosa* strain RP73 embedded agar beads at either  $1 \times 10^6$  or  $1 \times 10^5$  cfu/mouse using the method previously described by Bragonzi and colleagues [21]. Sham control mice were inoculated with sterile agar beads. 24 hours before infection, mice were treated intra-muscularly (*i.m*) with anti-GPIIb $\alpha$  platelet depleting or IgG control antibodies. Platelet numbers were enumerated in whole blood using an improved Neubauer haemocytometer to ensure sufficient platelet depletion.

### **Quantification of the platelet activation markers Platelet-Factor 4 (PF-4) and $\beta$ -thromboglobulin ( $\beta$ -TG) via ELISA**

24 hours post infection, plasma was isolated from citrated blood and BALF collected. Total levels of platelet derived PF-4 and  $\beta$ -TG were quantified from all samples and measured by ELISA according to manufacturer's protocols.

**Bronchoalveolar lavage collection and analysis**

24 hours post infection, BALF was collected. Cells were stained with Turk's nuclear stain and enumerated under a x20 objective using an Improved Neubauer haemocytometer. Cytospin slides were stained using Diff-Quick for differential cell count quantification.

**Lung, spleen and kidney harvest for microbiological and histological analysis**

24 hours post infection, colony forming units (CFUs) were quantified in lung, spleen and kidney homogenates. Lungs from separate mice were fixed in 10% formalin and embedded in paraffin blocks for histological analysis. Tissue sections were immunostained for platelets using a platelet specific anti-CD42b antibody, whilst additional sections were stained with an anti-neutrophil elastase antibody or with Haematoxylin and Eosin for visualisation of inflammatory cell infiltrate.

**Platelet quantification in BAL fluid**

Cytospins prepared from BAL fluid were immunostained for platelets using a platelet specific anti-CD41 antibody, a secondary mouse-adsorbed biotinylated anti-rat secondary antibody, and an avidin-biotin-HRP complex. Stained platelets were quantified by traversing the whole area of the cytospin under an x40 objective.

**Evaluation of biochemical markers of metabolic acidosis**



24 hours post infection with  $1 \times 10^5$  cfu/mouse *P. aeruginosa*, blood was collected via cardiac puncture into heparin coated syringes. Blood biochemical changes were measured using a handheld i-STAT blood analyser and EC8+ cartridges.

#### **Platelet-Bacterial Co-cultures.**

Primary overnight cultures of *P. aeruginosa* (RP73) were prepared and diluted to  $1 \times 10^6$  cfu/ml. Washed platelets were isolated from citrated blood from healthy male C57/Bl6 mice and diluted to  $1 \times 10^6$  platelets/ml. *P. aeruginosa* and platelets were incubated together at a 1:1 ratio and cultured at 37°C. Aliquots of the co-culture were taken periodically over 8 hours and plated for cfu quantification.

#### **Statistical Analysis**

Data from bacterial studies are expressed as mean  $\pm$  SEMs. Data were tested for normality using the Shapiro-Wilk normality test and analysed using a 1-way ANOVA, followed by Bonferroni's multiple comparison test. Weight data was analysed using a Kruskal-Wallis test followed Dunn's multiple comparison test. ELISA data was analysed using a Two-Tailed Mann Whitney T-test. Survival curves were analysed using a Mantel-Cox test. A *P* value of less than 0.05 was considered significant.

## RESULTS

### Circulating platelet numbers were suppressed following *P. aeruginosa* infection

A significant decrease in circulating platelet numbers was observed 24 hours post infection with *P. aeruginosa*. This was significant in mice infected with both  $1 \times 10^6$  cfu/mouse *P. aeruginosa* (Sham:  $6.8 \pm 0.4 \times 10^8$  platelets/ml vs. *P. aeruginosa*:  $5.9 \pm 0.5 \times 10^8$  platelets/ml, 13% decrease,  $P < 0.05$ , **Figure 1A**) and  $1 \times 10^5$  cfu/mouse *P. aeruginosa* (Sham:  $6.7 \pm 0.4 \times 10^8$  platelets/ml vs. *P. aeruginosa*:  $5.6 \pm 0.3 \times 10^8$  platelets/ml, 17% decrease,  $P < 0.05$ , **Figure 1B**). The occurrence of peripheral thrombocytopaenia after lung infection may be a consequence of localised pulmonary platelet recruitment due to platelet activation. We therefore investigated whether platelet activation and accumulation had occurred in the lungs 24 hours post infection.

### Pulmonary *P. aeruginosa* infection caused an increase in platelet-associated activation markers

Upon platelet activation, PF-4 and  $\beta$ -TG are secreted from internal granules. We therefore measured the levels of  $\beta$ -TG and PF-4 secretion as indicators of platelet activation in blood plasma and BALF. 24 hours post infection with *P. aeruginosa*, a significant increase in plasma  $\beta$ -TG levels was observed when compared to sham controls (Sham:  $73.63 \pm 6.11$  pg/ $\mu$ g protein vs. *P. aeruginosa* + IgG Control:  $103.51 \pm 8.83$  pg/ $\mu$ g protein,  $P < 0.05$ , **Figure 2A**).  $\beta$ -TG levels also appeared to be elevated in BAL fluid obtained 24 hours post infection (Sham:  $2.12 \pm 0.15$  pg/ $\mu$ g protein vs. *P. aeruginosa* + IgG Control:  $3.38 \pm 0.71$  pg/ $\mu$ g protein), although this increase did not reach statistical significance (**Figure 2B**).

No detectable changes in plasma PF-4 levels were observed 24 hours post infection (**Figure 2C**). However, at this timepoint PF-4 levels were significantly elevated in the BAL fluid suggesting that PF-4 may only be released by platelets at the site of infection (Sham:  $0.18 \pm 0.01$  OD vs. *P. aeruginosa*:  $0.31 \pm 0.05$  OD,  $P < 0.05$  **Figure 2D**).

### **Histological evidence for platelet accumulation in lung tissue following *P. aeruginosa* infection**

Lung samples collected 24 hours post infection, stained with an anti-neutrophil elastase antibody or with a H&E stain, indicated an increase in neutrophil recruitment into the airway lumen and alveolar spaces (**Figure 3A**). In these samples, an increase in platelet specific staining was also observed through specific anti-CD42b staining (**Figure 3B**). This staining was quantified and a significant increase in extravascular pulmonary platelet accumulation was observed in mice infected with *P. aeruginosa* compared to sham control mice (Sham:  $3568.33 \pm 457.53$  platelets/mm<sup>2</sup> vs. *P. aeruginosa*:  $9670 \pm 1457.06$  platelets/mm<sup>2</sup>,  $P < 0.01$ , **Figure 3C**). Regions positive for CD42b also appeared to be present around bacteria in the airways of *P. aeruginosa* infected mice (data not shown). However, further research is required to identify whether this is representative of direct platelet-bacterial interactions that have previously been observed *in vitro* [17]. Platelet recruitment into the airway lumen was subsequently confirmed using platelet specific anti-CD41 staining on slides prepared from isolated BALF, which when quantified demonstrated significant increases in platelets present in the lavage fluid of infected mice (Sham:  $0.30 \pm 0.16 \times 10^3$  platelets/ml vs. *P. aeruginosa*:  $2.64 \pm 0.58 \times 10^3$  platelets/ml,  $P < 0.01$ , **Figure 3D**).

### **Experimentally induced platelet depletion significantly decreased *P. aeruginosa* induced pulmonary leukocyte recruitment**

To understand whether the pulmonary accumulation of platelets in response to *P. aeruginosa* infection was a cause or consequence of the immune response to the invading pathogen, we conducted experiments in which mice were experimentally depleted of circulating platelets. To induce a sufficient decrease in circulating platelet numbers (> 80%), mice were treated with either an IgG control antibody or 10 $\mu$ M of the polyclonal anti-GPIb $\alpha$  platelet depleting antibody prior to infection with *P. aeruginosa*. This induced a significant state of platelet depletion in both studies (85%,  $P < 0.001$ , **Figure 1A & B**) which was maintained throughout the 24-hour study period. No effects of the anti-GP1b $\alpha$  platelet depleting antibody were observed on whole blood neutrophil counts (**Supplementary Figure 1B**).

Infection with  $1 \times 10^6$  cfu/mouse *P. aeruginosa* induced a significant increase in total pulmonary leukocyte recruitment ( $P < 0.05$ ) at 24 hours (**Figure 4A**). This was characterised by a significant increase in pulmonary neutrophil recruitment ( $P < 0.01$ ) (**Figure 4B**) as well as increases in both lymphocytes and macrophages, although these latter cell types did not achieve statistical significance (**Figure 4C+D**). Mice depleted of circulating platelets (>85%) demonstrated a reduction in *P. aeruginosa* induced total leukocyte recruitment, although this was not significant (**Figure 4A**). However, a significant reduction in neutrophil recruitment was observed ( $P < 0.05$ ) (**Figure 4B**) as well as a reduction in lymphocyte numbers, although this was not significant (**Figure 4C**). No discernible changes in pulmonary macrophage numbers were observed in mice depleted of circulating platelets (**Figure 4D**).

Similarly, in mice inoculated with  $1 \times 10^5$  cfu/mouse *P. aeruginosa*, total pulmonary leukocyte recruitment was significantly elevated ( $P < 0.001$ ), which was also characterised by a

significant increase in neutrophil recruitment ( $P<0.001$ ). In mice depleted of circulating platelets both total pulmonary leukocyte recruitment ( $P<0.01$ ) whilst both neutrophil ( $P<0.05$ ) and macrophage ( $P<0.05$ ) recruitment were significantly reduced (**Supplementary Figure 2**).

### **Bacterial colonisation is enhanced significantly in mice depleted of platelets.**

24 hours post infection, mice inoculated with sterile PBS embedded agar beads demonstrated no evidence of infection in the lungs, kidney or spleen. In contrast, mice infected with  $1 \times 10^6$  cfu/mouse and treated with an IgG control antibody demonstrated a significant increase in total pulmonary bacterial load ( $5.08 \pm 0.54$  log cfu/ml,  $P<0.001$ , **Figure 5A**), with minimal systemic infection observed in the kidneys and spleen (**Figure 5B+C**). However, in mice depleted of circulating platelets, pulmonary bacterial load was significantly increased compared to IgG control treated mice ( $6.62 \pm 0.35$  log cfu/ml,  $P<0.05$ ). This was described by an increase in pulmonary bacterial load of approximately 1.5 log cfu increase (**Figure 5A**). This increase was accompanied by an additional increase in bacterial numbers in both the kidney and spleen in mice depleted of platelets, although this did not reach statistical significance (**Figure 5B+C**). To investigate further the increase in the incidence of systemic infection, we performed further experiments to measure bacterial load 24 hours post infection using a lower inoculum of  $1 \times 10^5$  cfu/mouse *P. aeruginosa*. The primary objective of using a lower inoculum was to reduce the severity of the model as we had seen an increase in the incidence of mortality following infection with  $1 \times 10^6$  cfu/mouse. In animals infected with the lower inoculum of  $1 \times 10^5$  cfu/mouse *P. aeruginosa*, no infection was observed in the lungs, spleen of kidneys of mice inoculated with sterile agar beads

(**Figure 5D-F and Supplementary Figure 3A-C**). Mice treated with the IgG control antibody and infected with the lower inoculum of  $1 \times 10^5$  cfu/mouse *P. aeruginosa* also showed a significant elevation in total pulmonary bacterial load ( $4.72 \pm 0.39$  log cfu/ml,  $P < 0.001$ , **Figure 5D**). In contrast to the data collected from mice infected with  $1 \times 10^6$  cfu/mouse, mice depleted of platelets prior to infection with  $1 \times 10^5$  cfu/mouse *P. aeruginosa* did not show a significant increase in total bacterial load 24 hours post infection compared to IgG control antibody treated mice ( $5.21 \pm 0.39$  log cfu/ml). However, a small non-significant increase of 0.5 log cfu was observed in the lung. This could be explained by the lower inoculum producing a less severe infection within the lungs compared to the  $1 \times 10^6$  cfu/mouse inoculum. Similarly, to the studies using  $1 \times 10^6$  cfu/mouse, IgG control antibody treated infected mice demonstrated no systemic infection in the spleen and kidneys. In contrast, in mice depleted of platelets, we demonstrated a significant elevation in bacterial numbers in the kidney (log  $2.07 \pm 0.24$  cfu/ml,  $P < 0.001$ , **Figure 5E**) and the spleen (log  $0.74 \pm 0.36$  cfu/ml,  $P < 0.05$ , **Figure 5F**).

#### **Mice depleted of platelets infected with *P. aeruginosa* demonstrated increased mortality**

In mice infected with  $1 \times 10^6$  cfu/mouse *P. aeruginosa* we observed a significantly increased mortality rate (40%,  $P < 0.05$ ) in mice experimentally depleted of platelets compared with IgG control antibody treated mice (0%) (**Figure 6A**). Following infection with  $1 \times 10^5$  cfu/mouse, mice depleted of circulating platelets showed a decreased mortality rate compared to those infected with  $1 \times 10^6$  cfu/mouse *P. aeruginosa* with mortality levels of only 20%. However, this was still elevated when compared to mice infected with  $1 \times 10^5$  cfu/mouse which had received the IgG control antibody (0%) (**Figure 6B**). These data suggest that whilst the

infection profile with the lower inoculum was associated with decreased severity with no significant difference observed, the experimental depletion of circulating platelets continued to produce a trend suggesting a more severe phenotype. As increases in mortality were observed, we investigated the potential cause of death in these animals. Metabolic acidosis is a known biomarker of organ dysfunction in septic patients, and severe metabolic acidosis is indicative of multiple organ dysfunction syndrome (MODS)[23]. We therefore assessed blood biochemical markers associated with metabolic acidosis in animals following infection with  $1 \times 10^5$  cfu/mouse *P. aeruginosa*. In the mice that survived the 24-hour study duration, no significant differences were observed in blood Urea, pH, Base Excess  $\text{HCO}_3$ ,  $\text{pCO}_2$  levels and the Anion Gap. However, a trend towards a decrease in these parameters was observed in infected mice depleted of circulating platelets (**Supplementary Table 1**). Whilst these data did not show any significant differences, the subtle changes observed are consistent with metabolic acidosis and the absolute values and degree of the changes are consistent with previously published models of systemic inflammatory response syndrome (SIRS) and septic infection [24]. The shift in the systemic blood biochemistry observed in these animals may have been more pronounced in the animals that died prior to the 24-hour end point of the study, therefore further work is required to identify if metabolic acidosis leading to MODS is the primary cause of death in these animals.

### **Platelets Modulate Bacterial Growth kinetics *in vitro***

Our *in vivo* studies detected an increase in total bacterial load 24 hours post infection. Animals infected with the higher inoculum demonstrated a 1.5 log cfu increase in pulmonary bacterial load ( $5.08 \pm 0.54$  vs  $6.62 \pm 0.35$  log cfu). This was interesting as this

strain of *P. aeruginosa* has previously demonstrated a similar log cfu increase over the same time period in *in vitro* cultures [25]. We therefore investigated whether platelets had any effect on bacterial growth *in vitro*. Platelet bacteria co-culture experiments were performed and cfu's quantified over an 8-hour time course. Bacteria cultured in the presence of  $1 \times 10^6$ /ml platelets demonstrated significant decreases at both 6 (Bacteria alone:  $\log 8.49 \pm 0.22$  cfu/ml vs. Bacteria + Platelets:  $\log 7.74 \pm 0.42$  cfu/ml,  $P < 0.05$ ) and 8 hours (Bacteria alone:  $\log 8.91 \pm 0.19$  cfu/ml vs. Bacteria + Platelets:  $\log 7.80 \pm 0.28$  cfu/ml,  $P < 0.001$ ) when compared to bacterial cultures in the absence of platelets (**Figure 7**).

## DISCUSSION

Here we provide compelling evidence that platelets play an important role in the host response to pulmonary bacterial infection with *P. aeruginosa*. In our model, we have demonstrated that experimentally induced platelet depletion permits increased pulmonary bacterial growth and systemic bacterial dissemination, which is not normally associated with infections with this strain of *P. aeruginosa*. Our observations therefore support the hypothesis that platelets play a significant role in containing bacterial infections to the lung. Furthermore, we also provide evidence that platelets are required for neutrophil recruitment to the lung in an analogous manner to that described in other animal models of pulmonary leukocyte recruitment following both allergic and non-allergic inflammatory insults [6,7, 9].

Platelets (Murine and Human) variably express Toll-like Receptors (TLR) 2 and 4 on their surface [26]. TLR2 and TLR4 receptors recognise bacterial peptidoglycans and lipopolysaccharides (LPS) in gram-positive and gram-negative pathogens respectively [27–



30]. This provides a plausible mechanism for direct bacterial interactions with platelets which may contribute towards platelet activation. Indeed, platelet shape change, reversible states of platelet accumulation and adhesion, formation of platelet-leukocyte conjugates, elevated P-selectin expression and platelet granule release have all been detected following exposure to bacterial products or through direct interactions with bacterial pathogens, including *S.aureus*, *Streptococcus pyogenes*, *Escherichia Coli* and *Clostridium perfringens* [18,31,32].

Here we demonstrate that infection with  $1 \times 10^6$  cfu/mouse of *P. aeruginosa* resulted in increased BAL levels of platelet derived PF-4 and  $\beta$ -TG at 24 hours post infection. This supports bacterial induced platelet activation previously described in the literature [14–16,18,31,32]. We also observed significant increases in plasma  $\beta$ -TG levels, although no increases in plasma PF-4 were detected. This may suggest that PF-4 is only released at the site of infection. Furthermore, we have demonstrated platelet accumulation in the lung, in agreement with published literature demonstrating pulmonary platelet accumulation following exposure to bacterial products [33], coupled with that observed in response to both allergic and sterile inflammatory stimuli [9,34].

Our group has previously demonstrated an important role for platelets in leukocyte recruitment induced by both allergic and non-allergic inflammatory insults [5,6,8]. In the present study, we have extended these observations by showing an important role for platelets in pulmonary neutrophil recruitment following a bacterial infection, where we observed a significant decrease in neutrophil recruitment into the lungs in mice depleted of circulating platelets by greater than 85%. In our previous studies, using different inflammatory models we reported a more profound effect on pulmonary leukocyte

recruitment, this is perhaps due to the increased levels of platelet depletion (>95%), or to differences in the severity of the inflammatory response [5,6,8]. We have also recently demonstrated that pulmonary leukocyte recruitment following allergen challenge was P2Y<sub>1</sub> dependent [8]. This observation is of interest as P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors have been postulated to exert a protective role against infection with *P. aeruginosa* from observations of increased mortality and decreased pro-inflammatory cytokine release in P2Y<sub>1</sub>, P2Y<sub>2</sub> single and double knockout mice [35]. It is therefore plausible that platelet P2Y<sub>1</sub> receptor activation may be involved in pulmonary neutrophil recruitment in this lung infection model, similarly to that described for pulmonary cell recruitment either following allergen challenge or through exposure to LPS [8,36]

In addition to the engulfment of bacteria by neutrophils and the subsequent degradation of the pathogen through both oxidative and non-oxidative mechanisms, Neutrophil extracellular traps (NETs) are another mechanism which neutrophils can contribute to the killing and removal of bacteria from infected tissue [37–39]. The decreased pulmonary recruitment observed in our study in mice depleted of platelets is likely to contribute to the more severe phenotype observed as this will result in decreased capacity for neutrophil mediated bacterial trapping and killing [38,39]. However, our study only shows a partial inhibition of neutrophil recruitment, therefore there appears to be a component of neutrophil influx that is platelet independent following bacterial infection in the lung. Several studies have highlighted that platelets can induce NETosis through activation of neutrophils, either via direct interactions, release of mitochondria or via mitochondria containing platelet microparticles [37,40]. Clark and colleagues demonstrated that LPS stimulation, even at high concentrations, was insufficient to induce NETosis directly from neutrophils, and that platelet induced neutrophil activation is required for rapid LPS-

induced NETosis [28]. Whether this provides an additional mechanism by which platelets may be involved in the trapping and killing of bacteria in septic blood remains unclear and requires further study.

In the present study, pulmonary bacterial load in animals infected with  $1 \times 10^6$  cfu/mouse *P. aeruginosa* increased by 1.5 log cfu's over 24 hours in mice depleted of circulating platelets when compared to non-thrombocytopenic mice, whilst a more moderate 0.5 log cfu increase was observed following infection with the lower  $1 \times 10^5$  cfu/mouse inoculum. The significant decrease in neutrophil recruitment in platelet depleted mice is one mechanism by which to explain this increase in pulmonary bacterial load as neutrophils are essential in host defence towards bacterial infections through pathogen elimination. This has been shown through the susceptibility of neutropenic mice to this pathogen. However, it is interesting to note that the increased growth of bacteria ( $\sim 1.5$  log cfu/24 hours) in our model is similar to that observed for this strain in isolated *in vitro* cultures [25], suggesting that the platelets may contribute an additional component to the mechanisms restricting bacterial growth and survival *in vivo* further to neutrophil associated mechanisms. To this end, we performed bacterial growth curves both in the presence and absence of platelets to assess their role independent of neutrophils and demonstrated a significant reduction in viable bacteria at both 6 and 8 hours when cultured in the presence of platelets. This may indicate a mechanism that *in vitro*, may enable platelets to modulate bacterial growth kinetics independent of neutrophils, in addition to normal neutrophil involvement. The possibility of a direct mechanism of platelet modulation on bacterial growth is supported by work performed by Kramer and colleagues who demonstrated that the presence of platelets decreased the growth of *S. aureus in vitro* [41], and by the studies of De Stoppelaar *et al* who have demonstrated an increased bacterial burden in P-selectin deficient mice [16]. The

mechanism by which platelets limit bacterial growth is unclear, although work published from other laboratories has suggested that platelets have the capacity to directly internalise and phagocytose pathogens [14,42]. Whilst our *in vitro* findings highlight the possibility for a mechanism for platelet specific modulation of bacterial growth kinetics, the mechanisms behind this are beyond the scope of this study and further work is required to evaluate the mechanisms involved, and to what extent this is involved in *in vivo* models of infection.

The model of lung infection used in our study was adapted from a well-documented model of pulmonary infection with *P. aeruginosa* originally described by Cash and colleagues in 1979. This model has since been replicated by a number of independent research laboratories to investigate pulmonary infection in a number of species and is considered to be a non-systemic and non-fatal lung infection model [43]. However, in our studies following an inoculum of  $1 \times 10^6$  cfu/mouse *P. aeruginosa*, we observed a trend depicting an increase in the presence of systemic infection in the kidneys and spleen, and increased mortality (40%) in thrombocytopenic mice. When this study was repeated using a lower ( $1 \times 10^5$  cfu/mouse) inoculum to refine the model and decrease the severity we saw no evidence of systemic bacterial dissemination to the kidneys or spleen in mice with normal platelet levels. However, in mice experimentally depleted of circulating platelets we observed a significant increase in systemic bacterial numbers recovered from both the kidneys and the spleen. Whilst the mortality rates were lower than with the higher  $1 \times 10^6$  cfu/mouse inoculum, we still observed subtle increases in mortality (20%) in platelet depleted animals following infection with the lower  $1 \times 10^5$  cfu/mouse inoculum. Our observations suggested a conversion from a phenotype associated with localised infection to one displaying systemic infection as we demonstrated increased bacterial load in systemic organs, a subtle increase in biochemical markers of metabolic acidosis and

increased mortality. This suggests that platelets may play an important role in maintaining the integrity of the pulmonary vasculature to normally restrict pulmonary infections to the lung and preventing systemic bacterial dissemination towards distal organs. Indeed, others have shown that high circulating platelet numbers ( $5-13 \times 10^9$  platelets/litre) can prevent bleeding and offer protection against distal organ damage during gram-negative sepsis [15], whilst mice with platelets with impaired function through genetic deficiency of P-selectin demonstrate increased organ damage following *K. pneumoniae* infection [16]. We therefore suggest that in addition to normal neutrophil roles in bacterial infections, the removal of platelet mediated trapping and killing of bacteria through interactions with neutrophils, or through additional direct interactions with bacteria allows a transition from a mild to more severe infectious phenotype, supporting observations that worse clinical outcomes are associated with thrombocytopenia in patients with infections [11–13].

In conclusion, we have demonstrated that *P. aeruginosa* infection induces significant platelet activation and accumulation in lung tissue and BAL fluid 24 hours post infection. Furthermore, mice depleted of platelets demonstrated a 1.5 log cfu increase in pulmonary bacterial load, coupled with a similar increase in systemic bacterial load. Additionally, we observed a significant decrease in neutrophil recruitment following platelet depletion, which was associated with increased weight loss (**Supplementary figure 4**) and reduced survival rates from a strain of bacteria that is normally sub-lethal. We therefore conclude that in addition to the role that neutrophils play in the host response to bacterial infections, platelets also have distinct role in host defence against gram-negative infections in the lung.

## ACKNOWLEDGEMENTS

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## Figure Legends

**Figure 1. Pulmonary *P. aeruginosa* infection is associated with mild systemic thrombocytopenia.** Mice were treated with either 10 $\mu$ M anti-GPIIb $\alpha$  platelet depleting antibody (open circles) or IgG control antibody (filled circles) and infected with sterile PBS embedded agar beads and either 1x10<sup>6</sup> (A) or 1x10<sup>5</sup> (B) cfu/mouse *P. aeruginosa* embedded agar beads through intra-tracheal inoculation. 24 hours post infection, circulating platelet counts were enumerated from whole blood using a Zeiss axioscope microscope under a x40 objective. N=5-10. Data are expressed as mean  $\pm$  SEM, \* =p<0.05 compared to sham control mice, ### = P< 0.001 compared to *P.aeruginosa* + IgG control treated mice. Data was confirmed for Guassian distribution using the Shapira-Wilks normality test and subsequently analysed using one-way ANOVA and the Bonferroni multiple comparisons post test.

**Figure 2. *P. aeruginosa* infection is associated with increased release of the platelet derived mediators  $\beta$ -TG and PF-4.** Mice were infected with sterile PBS embedded agar beads and 1x10<sup>6</sup> cfu/mouse *P. aeruginosa* embedded agar beads through intra-tracheal inoculation. 24 hours post infection, plasma & BAL fluid were collected. ELISAs were performed on plasma and BAL fluid for quantification of the platelet derived mediators  $\beta$ -TG (A, B) and PF-4 (C,D). n = 4-7. Data are expressed as mean  $\pm$  SEM mice, \* = P< 0.05 compared to sham control mice. Data was confirmed for Guassian distribution using the Shapira-Wilks normality test and subsequently analysed using one-way ANOVA and the Bonferroni multiple comparisons post test.

**Figure 3. *P. aeruginosa* infection is associated with increased pulmonary leukocyte recruitment and pulmonary platelet accumulation *in vivo*.** Mice were infected with sterile PBS embedded agar beads and  $1 \times 10^6$  cfu/mouse *P. aeruginosa* embedded agar beads through intra-tracheal inoculation. 24 hours' post infection, lungs were removed and fixed in 10% PFA. Tissue sections were stained with neutrophils (neutrophil elastase), H&E and platelets (anti CD42b). **A:** Representative sections at x40 magnification of both parachemysa from sham and  $1 \times 10^6$  cfu/mouse *P. aeruginosa* infected mice for Neutrophil Elastase (i-ii) and H&E staining (iii-iv). **B:** Representative sections at x63 magnification of CD42b positive staining from sham (i-ii) and  $1 \times 10^6$  cfu/mouse *P. aeruginosa* (iii-iv) with red arrows indicating extravascular positive events. **C:** Quantification of CD42b positive staining in the tissue for analysis of platelet diapedesis. **D:** Quantification of platelet staining (CD41a) in BAL fluid from cytospin slides. n = 5-8 Data are expressed as mean  $\pm$  SEM \*\* =  $P < 0.01$  compared sham control mice. Data was confirmed for Guassian distribution using the Shapira-Wilks normality test and subsequently analysed using one-way ANOVA and the Bonferroni multiple comparisons post test.

**Figure 4. *P. aeruginosa* induced pulmonary leukocyte recruitment is reduced in platelet depleted mice.** Mice were treated with either 10 $\mu$ M anti-GPIb $\alpha$  platelet depleting antibody (open circles) or IgG control antibody (filled circles) and infected with sterile PBS embedded agar beads and  $1 \times 10^6$  cfu/mouse *P. aeruginosa* embedded agar beads through intra-tracheal inoculation. 24 hours post infection, BAL fluid was obtained and analysed for total cell counts (A), Neutrophils (B), Lymphocytes (C) and Macrophages (D). n=5-8. Data are expressed as mean  $\pm$  SEM. \* =  $P < 0.05$ , \*\* =  $P < 0.01$  compared to sham control mice, # =  $P < 0.05$  compared to IgG control infected *P. aeruginosa* mice. Data was confirmed for Guassian

distribution using the Shapiro-Wilks normality test and subsequently analysed using one-way ANOVA and the Bonferroni multiple comparisons post test.

**Figure 5. Depletion of circulating platelets is associated with increased pulmonary load and systemic dissemination of bacteria following infection with *P. aeruginosa*.** Mice were treated with either 10 $\mu$ M anti-GPIIb/IIIa platelet depleting antibody (open circles) or IgG control antibody (filled circles) and infected with sterile PBS embedded agar beads and 1x10<sup>6</sup> cfu/mouse (**A-C**) or 1x10<sup>5</sup> cfu/mouse (**D-F**) *P. aeruginosa* embedded agar beads through intra-tracheal inoculation. 24 hours post infection, the number of colony forming units (cfus) in Lung (**A & D**), Kidney (**B & E**) and Spleen homogenates (**C & F**) were quantified on TSA plates. n=5-10. Data expressed as Log mean  $\pm$  SEM. \* = P< 0.05, \*\*\* = P< 0.001 compared to sham control mice, # = P< 0.05, ### = P< 0.001 compared to IgG control infected *P. aeruginosa* mice, LOD = Limit of Detection. Data was confirmed for Gaussian distribution using the Shapiro-Wilks normality test and subsequently analysed using one-way ANOVA and the Bonferroni multiple comparisons post test.

**Figure 6. Depletion of circulating platelets is associated with increased mortality.** Mice were treated with either 10 $\mu$ M anti-GPIIb/IIIa platelet depleting antibody (blue triangle) or IgG control antibody (red square). Survival numbers were collected following infection with sterile PBS embedded agar beads and either 1x10<sup>6</sup> (**A**) or 1x10<sup>5</sup> (**B**) cfu/mouse *P. aeruginosa* embedded agar beads through intra-tracheal inoculation. n = 6-15. Survival plotted as survived = 0, dead = 1. \* = P< 0.05 compared to IgG control treated *P. aeruginosa* infected mice.

**Figure 7. Platelets reduce total bacterial numbers in culture *in vitro*.** *In vitro* cultures of  $1 \times 10^6$  cfu/ml *P. aeruginosa* were prepared alone (filled circle) or in the presence of  $1 \times 10^6$  platelets/ml (filled square). Aliquots were taken at 0, 2, 4, 6 and 8 hours and viable colony forming units quantified on TSA plates. n= 6. Data expressed as Log mean  $\pm$  SEM. \* =  $P < 0.05$ , \*\*\* =  $P < 0.001$  compared to bacterial control cultures. Data was confirmed for Gaussian distribution using the Shapira-Wilks normality test and subsequently analysed using one-way ANOVA and the Bonferroni multiple comparisons post test.

Figure 1

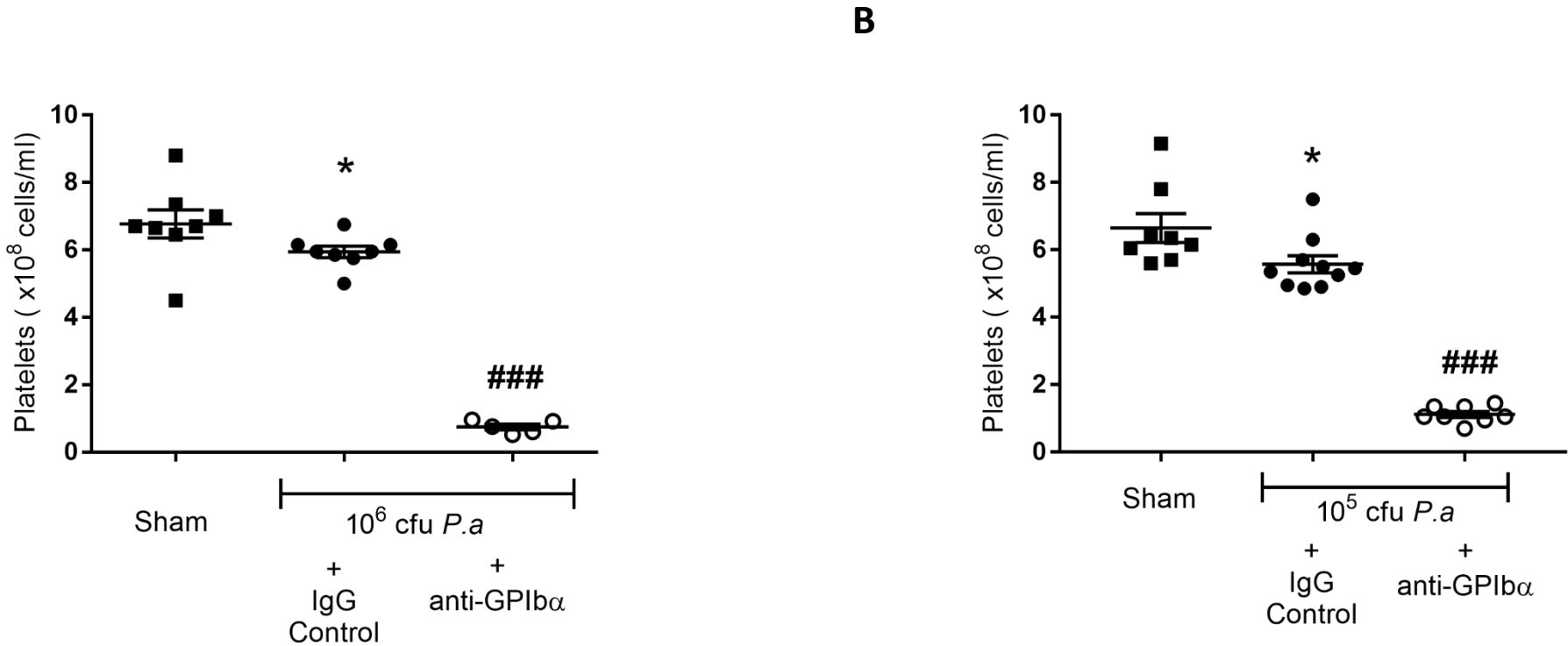
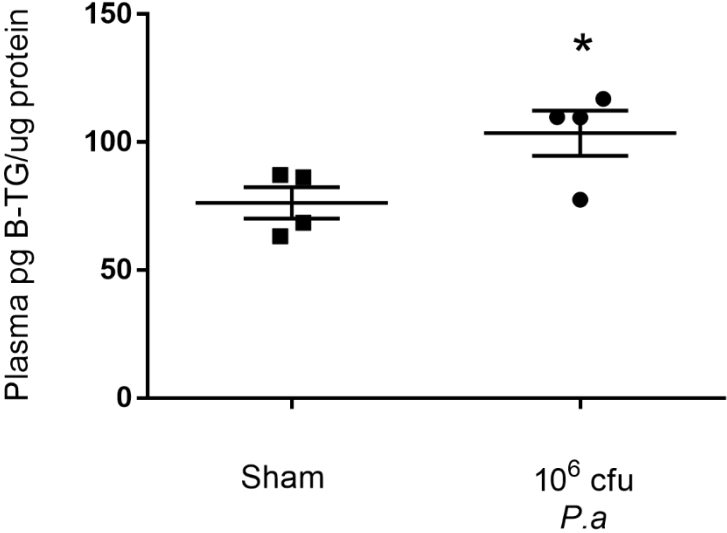


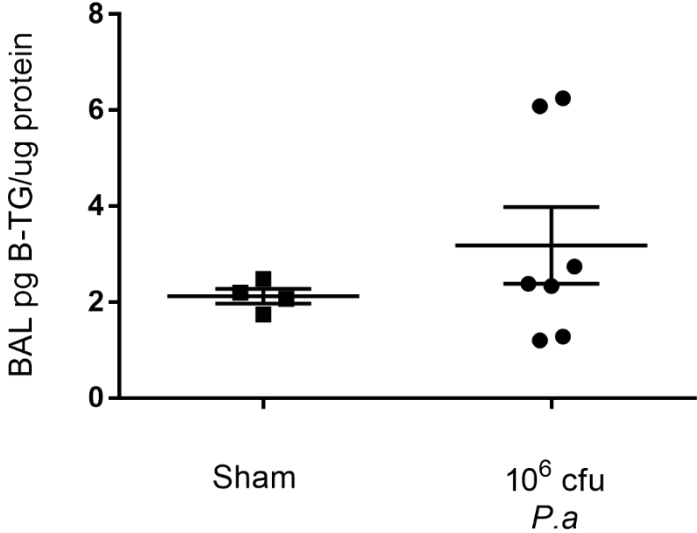


Figure 2

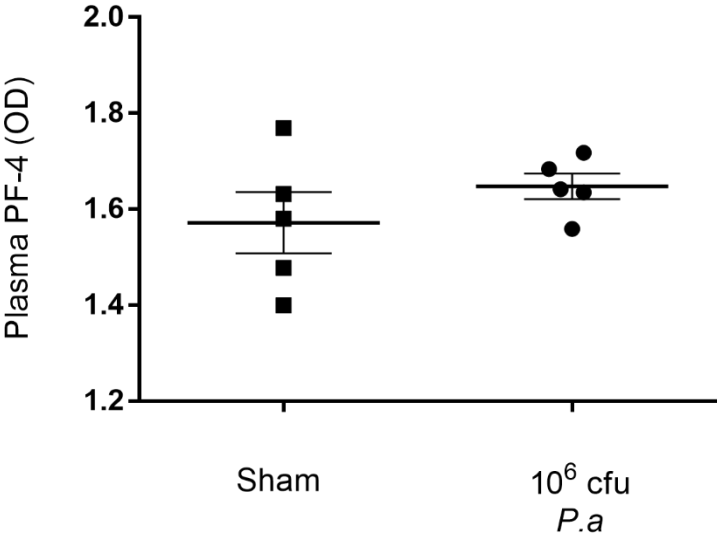
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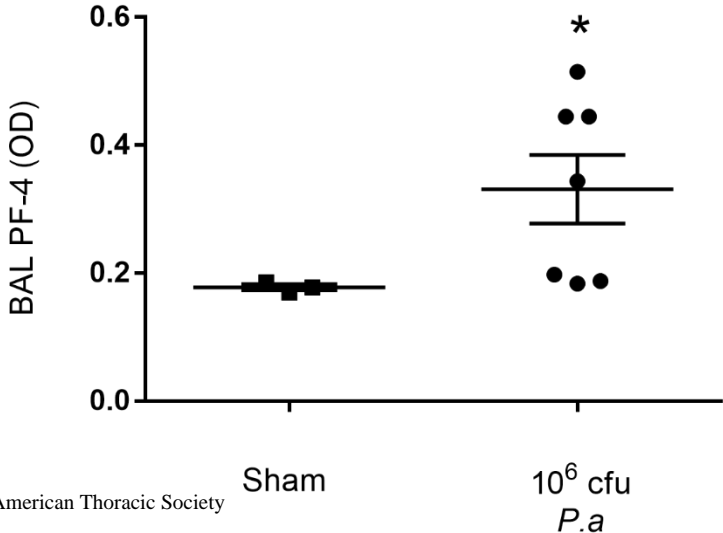
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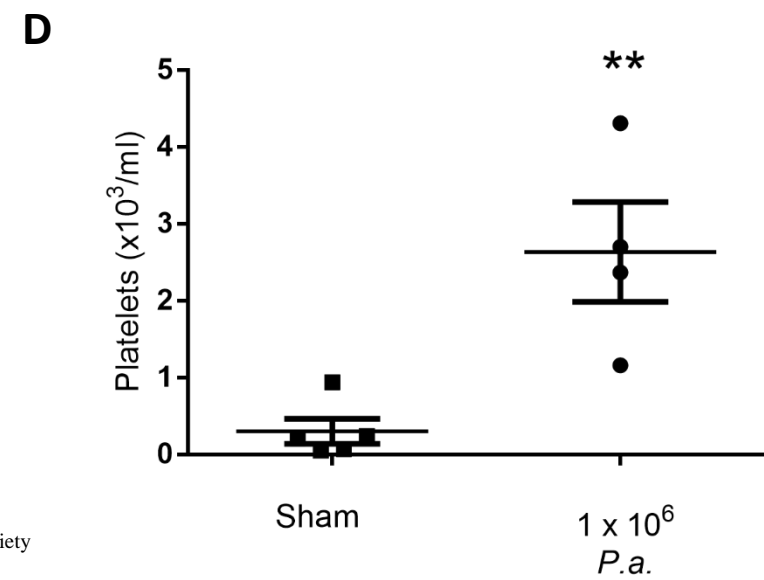
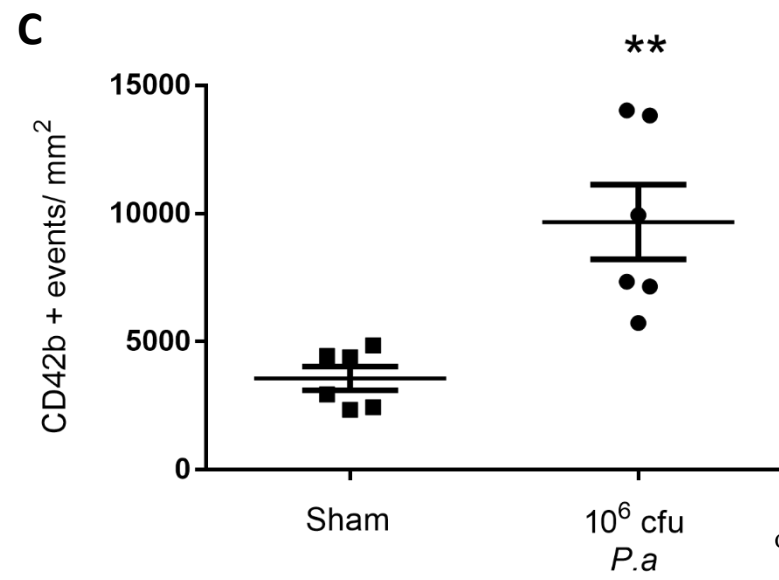
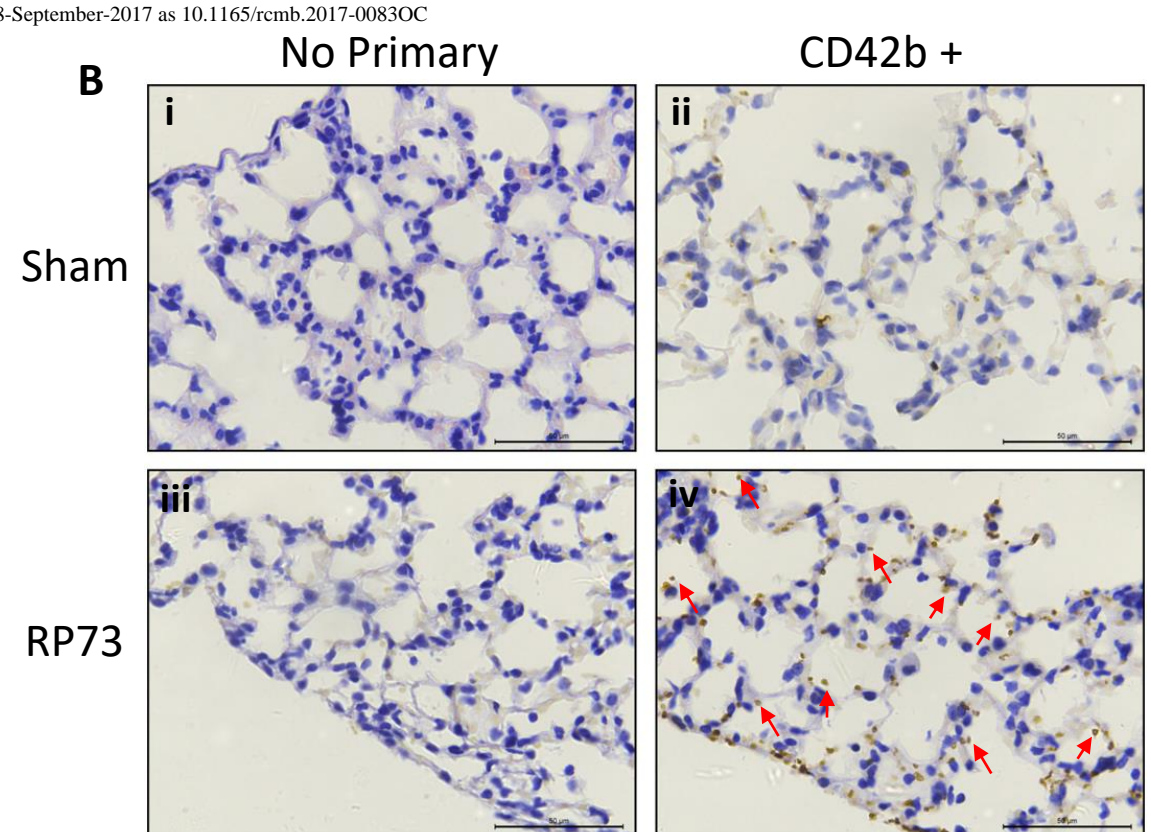
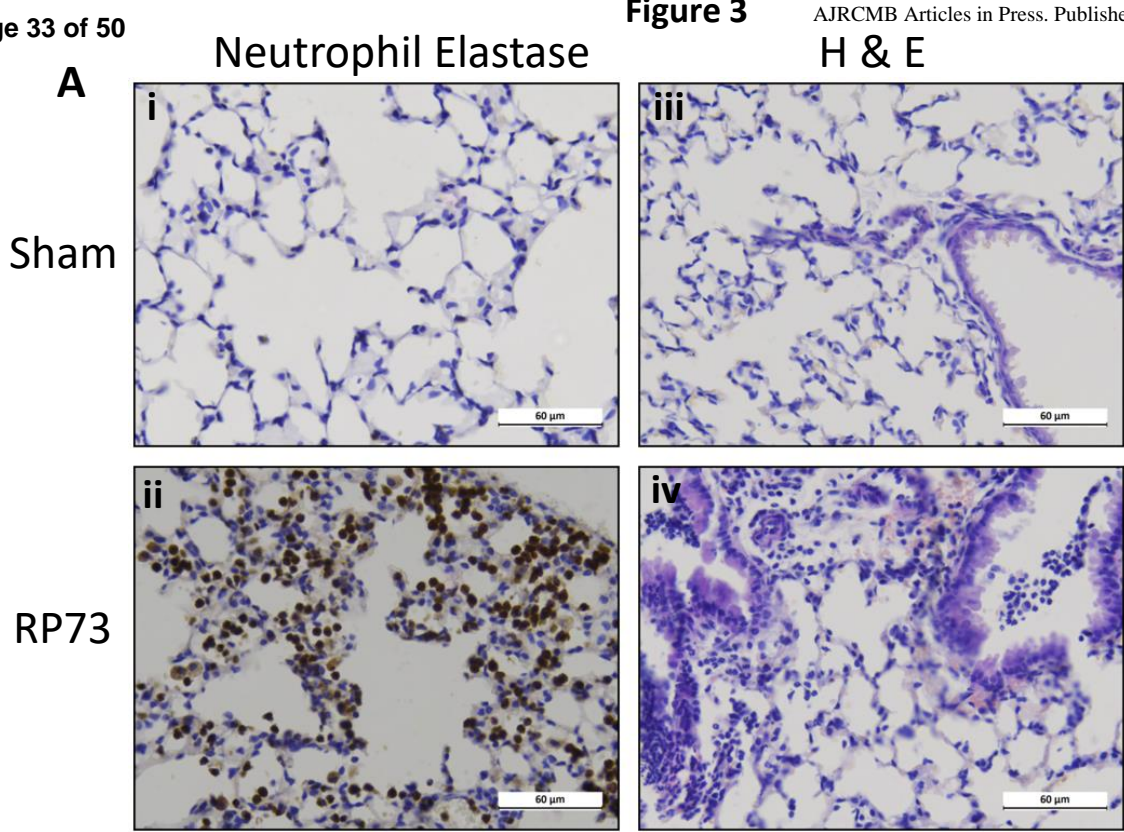


C



D





**Figure 4**

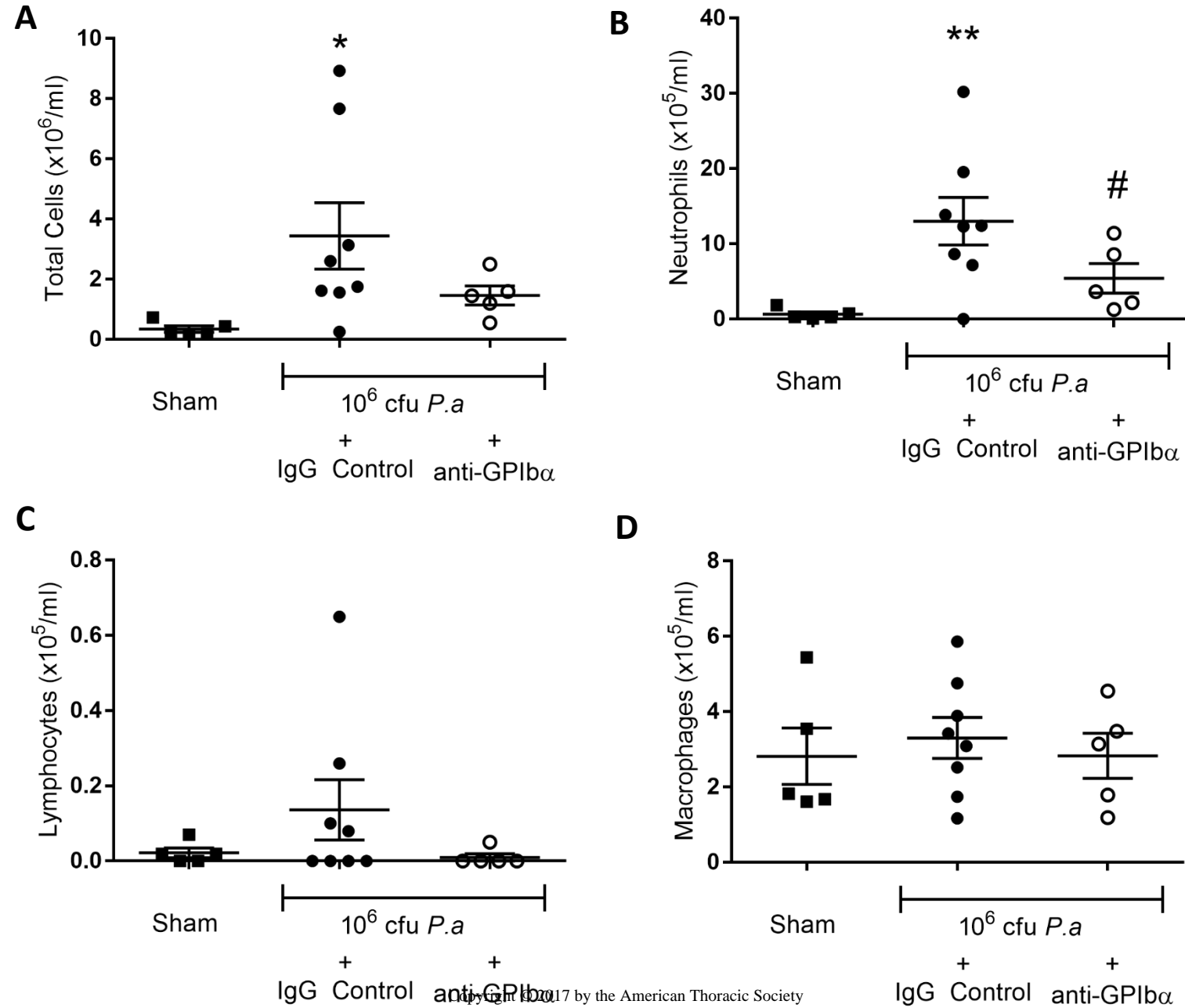


Figure 5

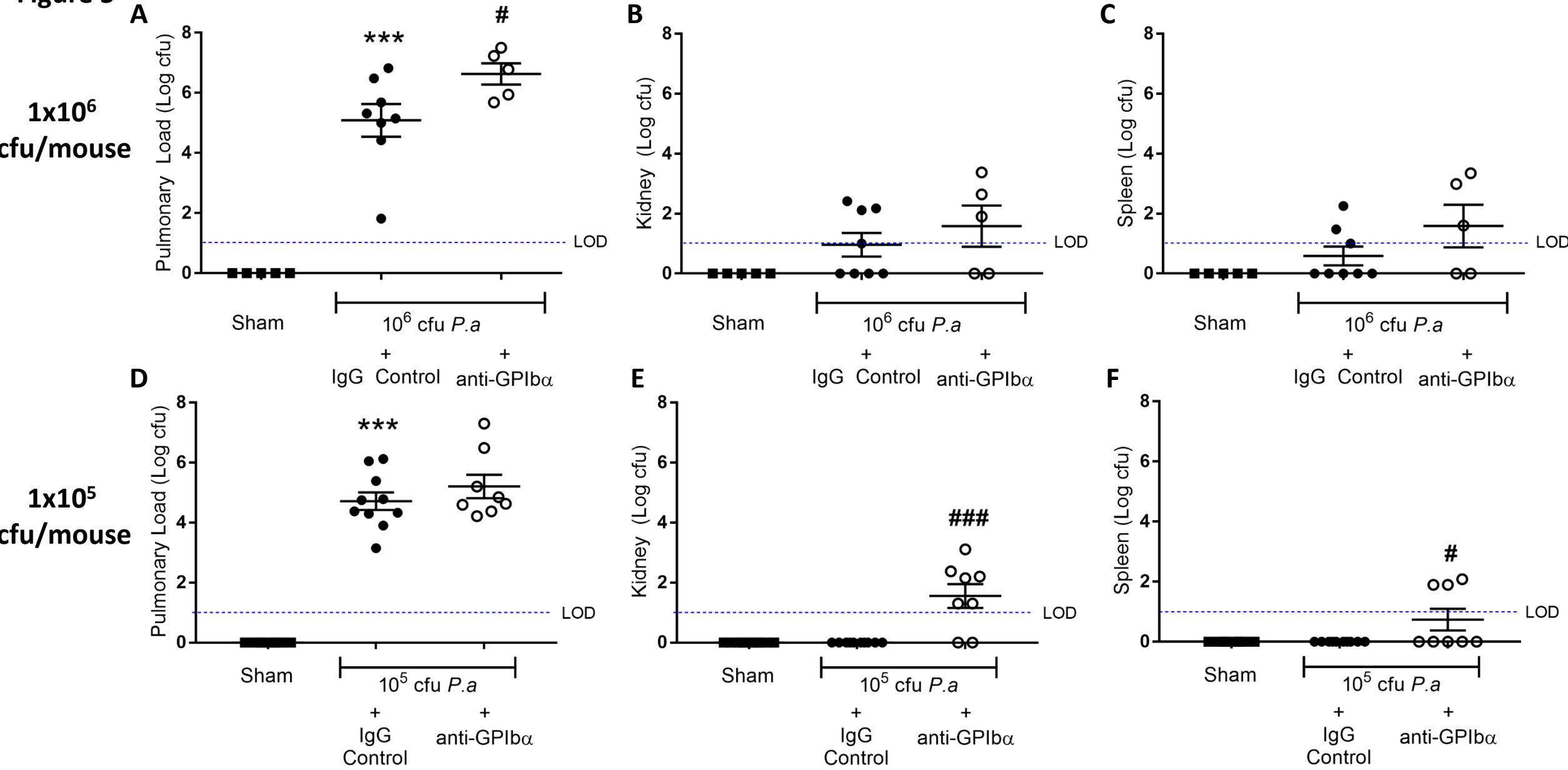


Figure 6

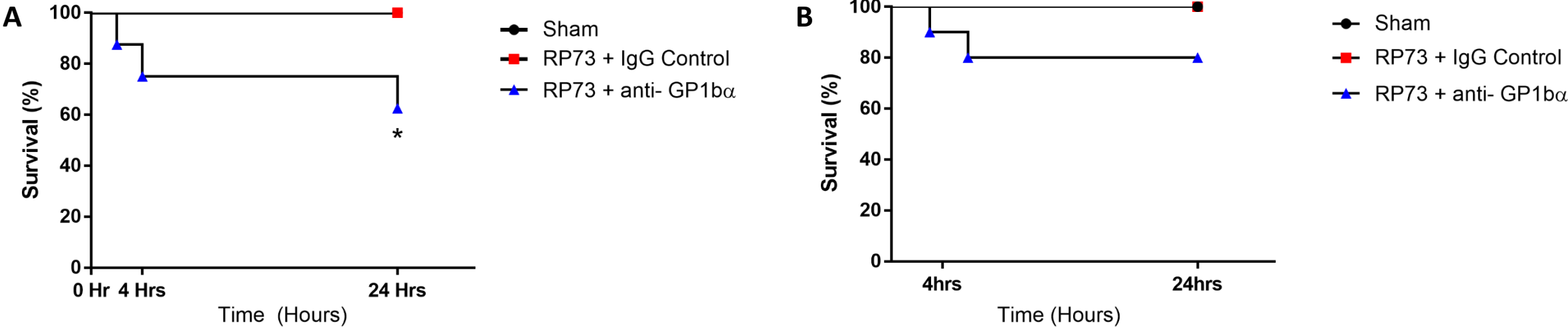
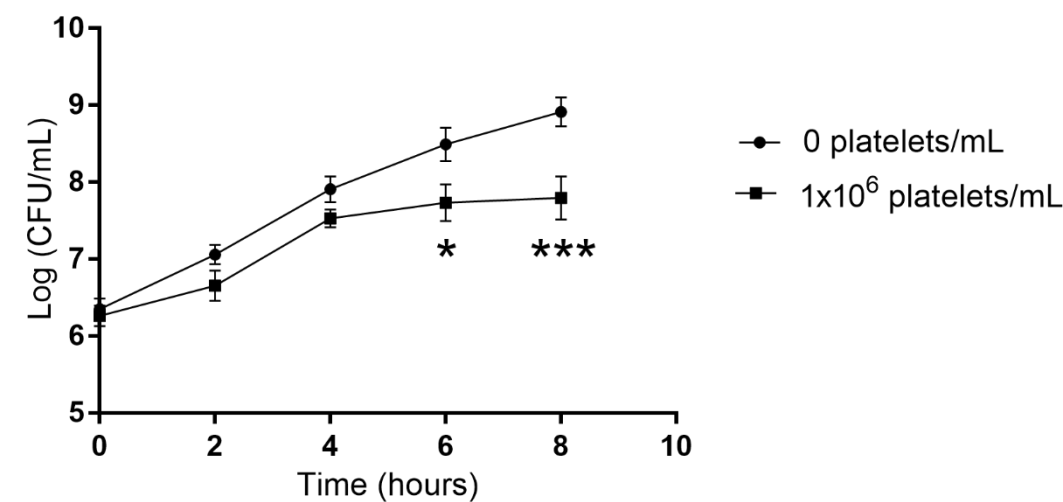


Figure 7



## SUPPLEMENTAL METHODS

### **Materials**

*P. aeruginosa* strain RP73 was obtained from a CF patient attending the Medizinische Hochschule of Hannover, Germany [20]. C57/Bl6 (8-10 weeks) male mice were obtained from Charles River, Italy or Harlan, UK. Other reagents were obtained from commercial suppliers: Tryptic Soy Agar (TSA), Trypticase Soy Broth, polyclonal anti-GPIIb $\alpha$  platelet depleting antibody and IgG control antibody, platelet specific anti-CD41 antibody, anti-CD42b antibody, PF-4 ELISA and a  $\beta$ -TG ELISA, secondary mouse-absorbed biotinylated anti-rat secondary antibody.

### **Preparation of agar beads embedded with *P. aeruginosa***

Using the method described by Bragonzi and colleagues [21], an overnight culture of *P. aeruginosa* strain RP73 was prepared in tryptic soy broth (TSB). This strain was chosen due to its lower acute virulence when compared to other *P. aeruginosa* strains, such as PAO1 [20], therefore reducing the expected severity of the lung infection following inoculation. *P. aeruginosa* strain RP73 was cultured in TSB overnight at 37°C, adjusted to a starting OD<sub>600nm</sub> of 0.025, and grown for additional 4 h for agar beads preparation. Bacteria were embedded into agar beads by mixing the overnight culture with molten tryptic soy agar, which was then spun into warmed mineral oil. The preparation was cooled and centrifuged at 2,700 g, the remaining oil was eliminated and the beads were washed in sterile PBS. The colony forming unit (cfu) content of the beads slurry was subsequently quantified on TSA plates

and the beads slurry then diluted to either  $2 \times 10^7$  cfu/ml or  $2 \times 10^6$  cfu/ml in sterile PBS to deliver a final dose of either  $1 \times 10^6$  or  $1 \times 10^5$  cfu/mouse.

### **Infection with *P. aeruginosa* and induction of platelet depletion in mice**

Animal work was conducted according to protocols approved by San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals. For studies using the  $1 \times 10^6$  cfu/mouse inoculum on day 0, male C57Bl6 mice were temporarily anesthetized by intraperitoneal injection (*i.p.*) of 2.5 mg of 2,2,2-tribromethanol (Avertin, Sigma) in 0.9% NaCl and administered in a volume of 15  $\mu$ l/g of body weight. The trachea was cannulated and a 50 $\mu$ l inoculum of bacteria embedded in agar beads ( $10^6$  cfu/mouse) was instilled directly into the respiratory tract. The trachea was cannulated with a sterile flexible 22-gauge catheter (Becton Dickinson, Madrid, Spain). Sham control mice were inoculated with sterile PBS agar beads. Body weight was measured daily and animals monitored at regular intervals for signs of pain and distress.

Additional studies using an inoculum of  $1 \times 10^5$  cfu/mouse were performed in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom). On day 0, male C57Bl6J mice were anesthetized under Isoflurane and 50 $\mu$ l inoculum of bacteria embedded in agar beads ( $10^5$  cfu/mouse) was instilled directly into the respiratory tract via oropharyngeal dosing (*o.a*) to reduce severity associated with surgery. Sham control mice were inoculated with sterile PBS agar beads. Body weight was measured daily and animals monitored at regular intervals for signs of pain and distress.



24 hours prior to infection, mice were treated intra-muscularly (i.m) with either 10 $\mu$ M of the polyclonal anti-GPIb $\alpha$  platelet depleting antibody (#R300, Emfret Analytics) or an IgG control antibody. Circulating platelet numbers were enumerated from whole blood collected via tail clips in stromatol on an improved Neubauer haemocytometer 24 hours post infection to ensure sufficient platelet depletion (>80% depletion).

### **Lung, spleen and kidney harvest for microbiological and histological analysis**

24 hours post infection, animals were terminally euthanized with a mix to O<sub>2</sub> 50%/CO<sub>2</sub> 50% or 25% Urethane. Lungs, kidneys and spleens were removed and homogenized in 2ml PBS. Samples were serially diluted 1:10 in PBS and plated for CFU count. Lung, kidney and spleen homogenates were then centrifuged at 14000 rpm for 30 min at 4°C and the supernatants were stored at -80°C for cytokine analysis.

For additional mice, lungs were inflated with 10% (v/v) buffered formalin and immersion fixed for 48 hours. Post fixation, lungs were sliced horizontally and processed into paraffin embedded blocks. De-waxed slices (5 $\mu$ m thick) prepared on a microtone were subsequently stained with a routine haematoxylin and eosin stain or for neutrophil elastase to permit histological assessment. Additional slides were stained for platelets using a platelet specific anti-CD42b antibody (#ab183345, abcam), a secondary mouse-adsorbed biotinylated anti-rat rabbit secondary antibody (Vector, BA-1000, 1:200), and an ABC Vectastain kit to assess platelet recruitment into different anatomical regions of the lung.

**Bronchoalveolar lavage fluid collection and analysis (total and differential cell count)**

A 22-gauge catheter was inserted into the trachea and BAL was recovered by instillation of 1 ml of RPMI 1640 three times and total cells counts were performed by adding Turks stain in a 1:1 ratio. Total cells were then enumerated on an improved Neubauer haemocytometer. BAL fluid were then centrifuged and supernatants were collected for quantification of total protein content.

Cytospin slides were prepared and 150ul of BAL was added into a cytospin funnel and centrifuged at 960rpm for 5 minute on a medium acceleration. The slides were then stained using Diff-Quick (Dade, Biomap, Italy). In addition, whole blood was diluted 1/100 in stromatol stain and circulating platelet numbers were quantified using an improved Neubauer haemocytometer under an x40 objective.

**Platelet quantification in BAL fluid**

The collected BALF was mixed and an aliquot removed and added into a cytospin funnel and centrifuged at 1000rpm for 1 minute on a slow acceleration. The slides were then stained with a primary anti-CD41 antibody (#SC-6604, Santa Cruz) and a secondary mouse-absorbed biotinylated anti-rat secondary antibody. Using an avidin-biotin-HRP complex, platelets in the BALF were quantified.

**Quantification of the release of the platelet activation markers PF-4 and  $\beta$ -TG via ELISA**

24 hours post infection, serum was isolated from citrated whole blood and BAL fluid. Total levels of platelet released PF-4 and  $\beta$ -TG were quantified from all samples and were measured by ELISA according to the manufacturer (R&D systems).

**Evaluation of biochemical markers of metabolic acidosis**

24 hours post infection with  $1 \times 10^5$  cfu/mouse *P. aeruginosa*, blood was collected via cardiac puncture into heparin coated syringes. 65 $\mu$ l blood was transferred to EC8+ blood analyser cartridges (Point of Care Testing Ltd: AB-3P7925) and processed using a handheld i-STAT blood analyser to quantify a panel of biochemical markers including pH, Urea, Base Excess, Bicarbonate ions, pCO<sub>2</sub> and Anion Gaps.

## Supplementary Legends

**Supplementary Figure 1. anti-GPIb $\alpha$  antibody depletes circulating platelet in sham infected mice.** Mice were treated with either 10 $\mu$ M anti-GPIb $\alpha$  platelet depleting antibody (open symbols) or IgG control antibody (filled symbols) and infected with either sterile PBS embedded agar beads (squares) or 1 $\times$ 10<sup>5</sup> cfu/mouse *P. aeruginosa* (circles) through intra-tracheal inoculation. 24 hours post infection, circulating platelet count (**A**) and whole blood neutrophil counts (**B**) were enumerated from whole blood using a Zeiss axioscope microscope under a x40 objective. N=8. Data are expressed as mean  $\pm$  SEM, \*\*\* =p<0.001 compared to sham IgG treated control mice. Data was confirmed for Guassian distribution using the Shapira-Wilks normality test and subsequently analysed using one-way ANOVA and the Bonferroni multiple comparisons post test.

**Supplementary Figure 2. *P. aeruginosa* induced pulmonary leukocyte recruitment is reduced in platelet depleted mice.** Mice were treated with either 10 $\mu$ M anti-GPIb $\alpha$  platelet depleting antibody (open circles) or IgG control antibody (filled circles) and infected with sterile PBS embedded agar beads and 1 $\times$ 10<sup>5</sup> cfu/mouse *P. aeruginosa* embedded agar beads through intra-tracheal inoculation. 24 hours post infection, BAL fluid was obtained and analysed for total cell counts (**A**), Neutrophils (**B**) and Macrophages (**C**). n = 4-6. Data are expressed as mean  $\pm$  SEM. \*\*\* = P< 0.001 compared to sham control mice, # = P< 0.05, ## = P < 0.01 compared to IgG control infected *P. aeruginosa* mice. Data was confirmed for Guassian distribution using the Shapira-Wilks normality test and subsequently analysed using one-way ANOVA and the Bonferroni multiple comparisons post test.

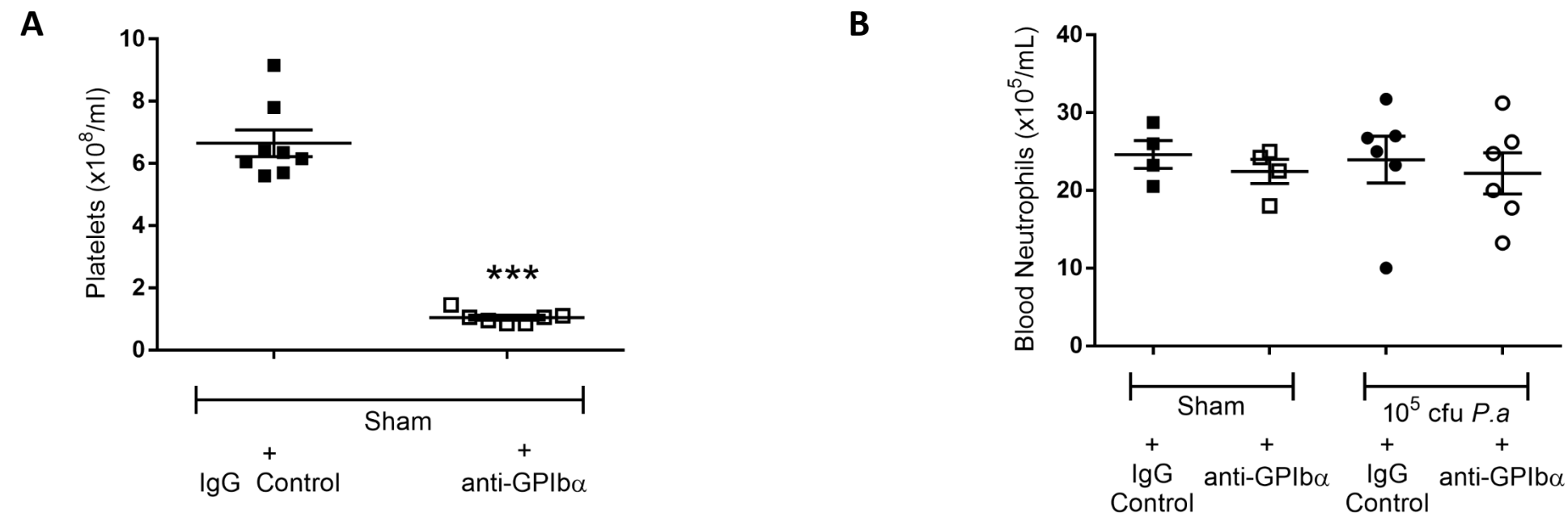
**Supplementary Figure 3. Effects of platelet depletion on bacterial numbers in sham infected mice.** Mice were treated with either 10 $\mu$ M anti-GPIb $\alpha$  platelet depleting antibody (open squares) or IgG control antibody (filled squares) and infected with sterile PBS embedded agar beads through intra-tracheal inoculation. 24 hours post infection, the number of colony forming units (cfus) in Lung (A), Kidney (B) and Spleen homogenates (C) were quantified on TSA plates. n=8-10. Data expressed as Log mean  $\pm$  SEM, LOD = Limit of Detection.

**Supplementary Figure 4. Pulmonary *P. aeruginosa* infection is associated with increased weight loss.** Mice were treated with either 10 $\mu$ M anti-GPIb $\alpha$  platelet depleting antibody (open circles) or IgG control antibody (filled circles) and infected with sterile PBS embedded agar beads and either 1 $\times$ 10<sup>6</sup> (A) or 1 $\times$ 10<sup>5</sup> (B) cfu/mouse *P. aeruginosa* embedded agar beads through intra-tracheal inoculation. Mice were weighed daily for the duration of the study and weight loss over time calculated, n= 4-10. Data expressed as mean  $\pm$  SEM. \*\* = P< 0.01 compared to sham control mice. Data was analysed using the Kruskal-Wallis test of variance followed by the Bonferonni multiple comparison post-test.

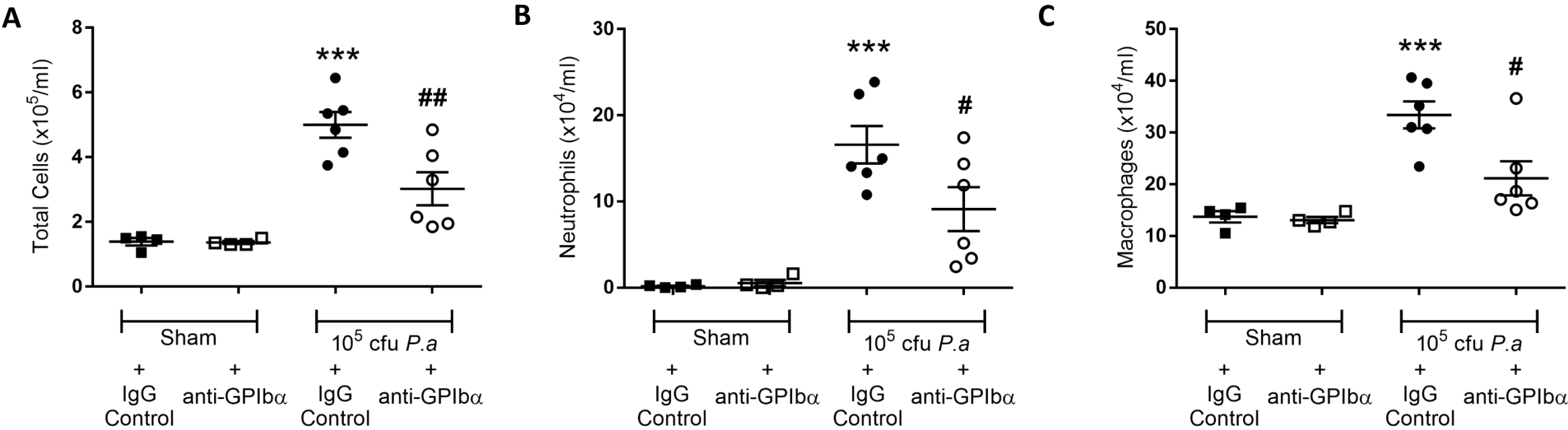
**Supplementary Table 1. Depletion of circulating platelets in infected mice is associated with biochemical markers of metabolic acidosis.** Mice were treated with either 10 $\mu$ M anti-GPIb $\alpha$  platelet depleting antibody or IgG control antibody and infected with sterile PBS embedded agar beads or 1 $\times$ 10<sup>5</sup> cfu/mouse *P. aeruginosa* embedded agar beads through intra-tracheal inoculation. 24 hours post infection, blood was collected via cardiac puncture

and blood biochemical markers were measured using EC8<sup>+</sup> blood analyser cartridges on an i-STAT handheld blood analyser, n = 4-6. Data expressed as mean  $\pm$  SEM.

Supplementary Figure 1



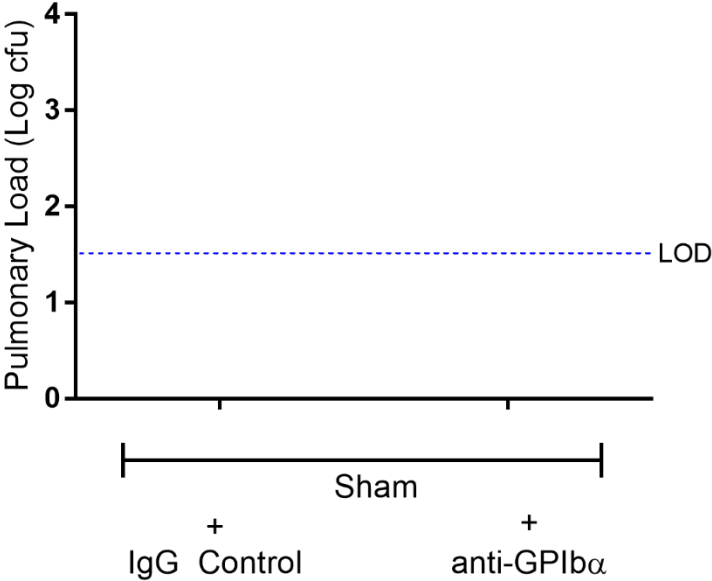
Supplementary Figure 2



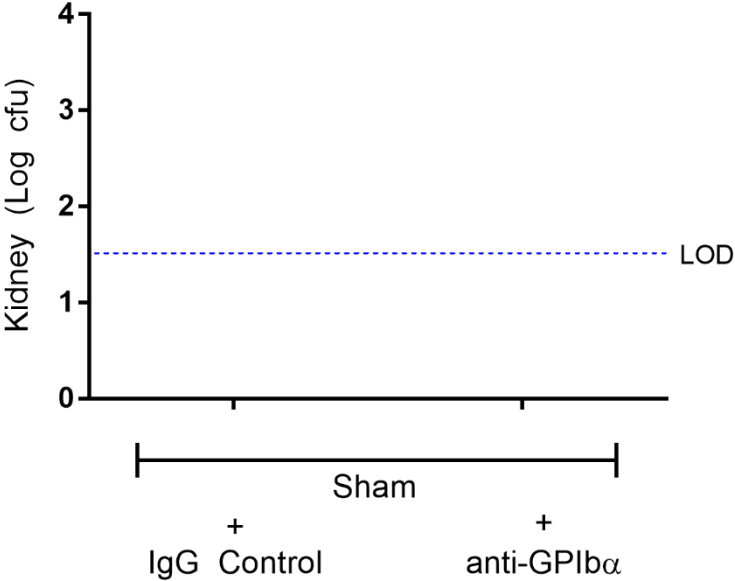


Supplementary Figure 3

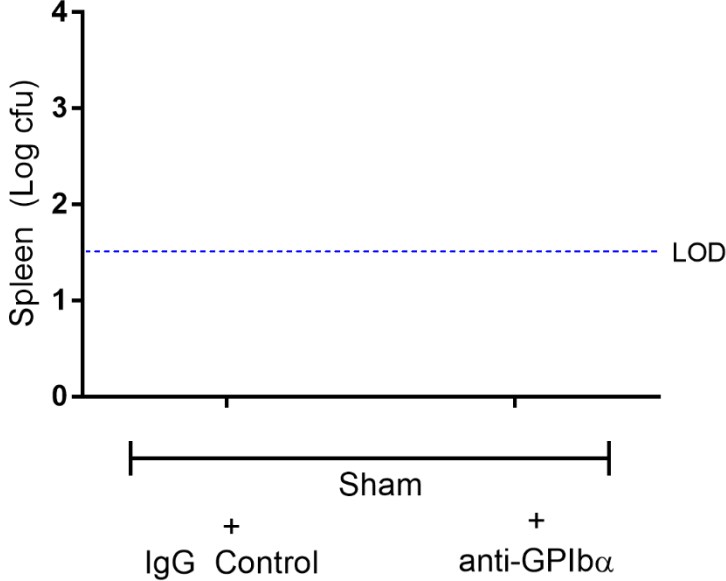
A



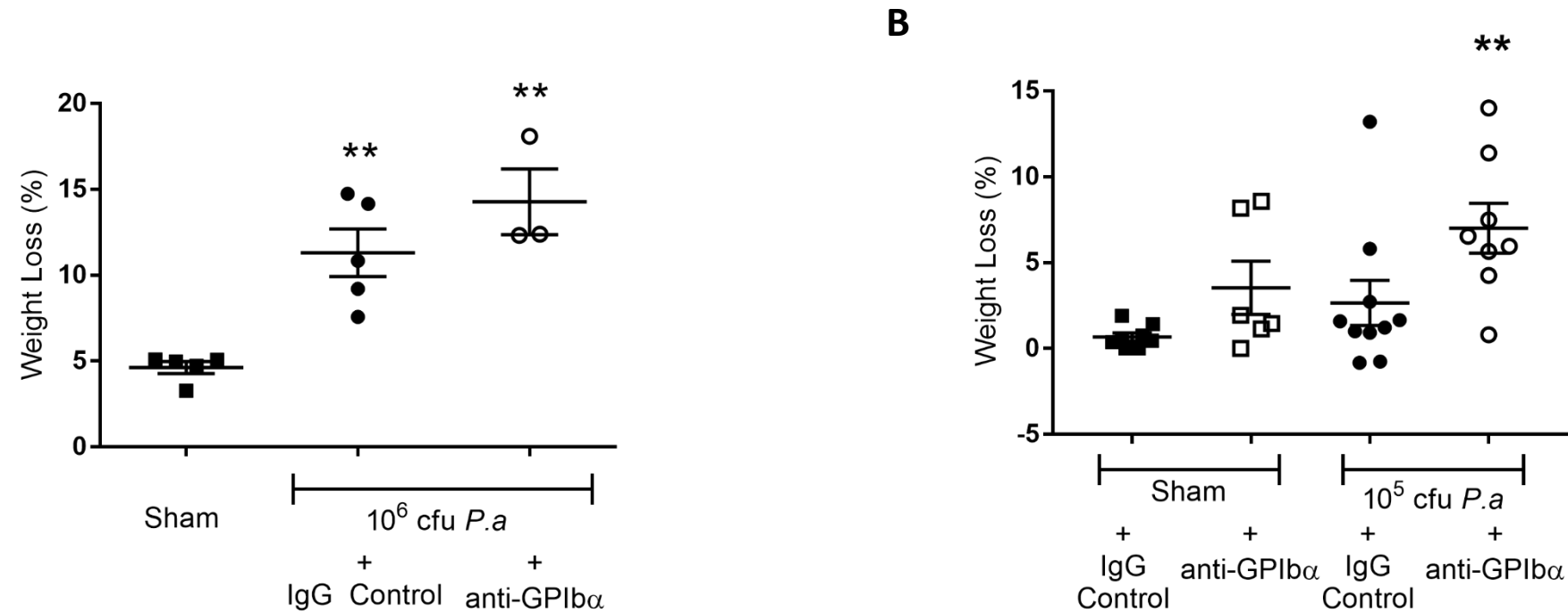
B



C



Supplementary Figure 4



**Table 1.** Blood Biochemistry in sham and *P.aeruginosa* infected mice

	Sham IgG Control	Sham anti-GPIIb $\alpha$	<i>P.aeruginosa</i> IgG Control	<i>P.aeruginosa</i> Anti-GPIIb $\alpha$
Urea, mmol/L	5.60 $\pm$ 0.38	5.80 $\pm$ 0.05	5.72 $\pm$ 0.37	9.08 $\pm$ 3.32
pH	7.14 $\pm$ 0.03	7.12 $\pm$ 0.01	7.15 $\pm$ 0.03	7.07 $\pm$ 0.04
Base excess, mmol/L	-12.00 $\pm$ 2.35	-14.03 $\pm$ 0.58	-12.88 $\pm$ 0.91	-16.61 $\pm$ 1.49
HCO <sub>3</sub> <sup>-</sup> , mmol/L	16.38 $\pm$ 1.92	14.13 $\pm$ 0.58	16.58 $\pm$ 0.87	12.91 $\pm$ 1.04
Glucose, mmol/L	12.45 $\pm$ 1.38	12.50 $\pm$ 1.01	11.27 $\pm$ 0.71	10.25 $\pm$ 0.79
Na <sup>+</sup> , mmol/L	146.00 $\pm$ 0.41	145.67 $\pm$ 0.58	145.67 $\pm$ 0.61	145.33 $\pm$ 0.84
K <sup>+</sup> , mmol/L	3.83 $\pm$ 0.34	4.43 $\pm$ 0.10	4.38 $\pm$ 0.30	4.93 $\pm$ 0.51
Cl <sup>-</sup> , mmol/L	116.00 $\pm$ 1.78	119.67 $\pm$ 0.76	115.83 $\pm$ 1.08	121.67 $\pm$ 1.43
PCO <sub>2</sub> mmHg	49.18 $\pm$ 2.71	44.37 $\pm$ 1.38	48.90 $\pm$ 2.56	41.15 $\pm$ 3.07
TCO <sub>2</sub> , mmol/L	18.50 $\pm$ 2.10	15.33 $\pm$ 0.76	18.16 $\pm$ 0.98	14.33 $\pm$ 1.24
AnGAP, mmol/L	17.25 $\pm$ 0.85	16.67 $\pm$ 0.58	17.33 $\pm$ 0.56	15.5 $\pm$ 0.72
Hct, %PCV	38.00 $\pm$ 2.00	19.5 $\pm$ 3.18	37.67 $\pm$ 1.20	19.00 $\pm$ 1.15
Hb, g/dL	12.93 $\pm$ 0.68	6.65 $\pm$ 1.10	12.80 $\pm$ 0.41	6.45 $\pm$ 0.39

Values are means  $\pm$  SEM; n = 4-6 mice. HCO<sub>3</sub><sup>-</sup>, bicarbonate; PCO<sub>2</sub>, partial pressure CO<sub>2</sub>; TCO<sub>2</sub>, total CO<sub>2</sub>; AnGAP, anion gap; Hct, Hematocrit; Hb, Haemoglobin; PCV, Packed Cell Volume